



(19) Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) Publication number:

0 640 689 A2

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: 94109773.5

(51) Int. Cl.: C12N 15/24, C12P 21/00,
C12N 5/06, A61K 38/22

(22) Date of filing: 24.06.94

(30) Priority: 02.07.93 US 87832

(71) Applicant: F. HOFFMANN-LA ROCHE AG
Grenzacherstrasse 124
CH-4002 Basel (CH)(40) Date of publication of application:
01.03.95 Bulletin 95/09(72) Inventor: Gately, Maurice Kent
162 Konner Avenue
Pine Brook,
New Jersey 07058 (US)
Inventor: Haldmi, John
210 Country Ridge Road
Scarsdale,
New York 10583 (US)
Inventor: Ling, Ping
293 Chestnut Street
Nutley,
New Jersey 07110 (US)(32) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IE IT LI LU MC
NL PT SE(74) Representative: Mezger, Wolfgang, Dr. et al
Grenzacherstrasse 124
Postfach 3255
CH-4002 Basel (CH)(54) **p40 Homodimer of interleukin-12.**

(57) The present invention is directed towards a protein comprising two p40 subunits of interleukin-12 which are associated together, preferably by at least one disulfide bond, having a molecular weight of about 80 kDa. The 80 kDa p40 homodimer acts as an interleukin-12 receptor antagonist. The preferred p40 subunit is that of SEQ ID NO:1.

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The present invention relates to a protein of two associated p40 subunits of interleukin-12 acting as an interleukin-12 receptor antagonist.

Interleukin-12 (IL-12), formerly known as cytotoxic lymphocyte maturation factor (CLMF) or natural killer cell stimulatory factor (NKSF), is a cytokine that has pleiotropic activities including stimulation of the proliferation of activated T and NK cells (1, 2), induction of INF- γ production by peripheral blood mononuclear cells and enhancement of the lytic activity of NK/LAK cells (2-4).

IL-12 is a heterodimeric molecule with an approximate molecular weight of about 75 kD consisting of two disulfide-linked subunits: p35, having an approximate molecular weight of about 35 kD, and p40, having an approximate molecular weight of about 40 kD, (2, 4-6). The p40 subunit shares amino acid sequence homology with the interleukin-6 receptor (IL-6R) and therefore belongs to the cytokine receptor superfamily, whereas p35 has a distant but significant relationship to the IL-6/G-CSF cytokine family. It has been speculated that the p35/p40 heterodimer could represent a cytokine (p35) and soluble cytokine receptor (p40) complex, with the cellular IL-12 receptor providing function analogous to the IL-6 signal transducing protein, gp130 (7, 8).

The biological activity of IL-12 is mediated by the binding of the intact IL-12 molecule to plasma membrane receptors on activated T or NK cells (9,10); however, the contributions of the individual subunits to receptor binding and signal transduction remain unknown. Studies with neutralizing antibodies to human IL-12 (11) and site-specific chemical modification (12) suggested that the p40 subunit contains epitopes important for IL-12 binding to its receptor. Also, studies with human/mouse chimeric molecules indicated that p35 is responsible for the species specificity of the heterodimer for biological activities.

Brief description of the drawings:

Figure 1. Dose-response binding of human IL-12 and COS-expressed rp40 to KIT225/K6 cells analyzed by flow cytometry. Varying concentrations of purified human IL-12 or rp40-containing conditioned medium (determined by EIA (enzyme immunoassay) using IL-12 as standard) were incubated with KIT225/K6 cells and detected with biotinylated 8E3 mAb followed by streptavidin-PE as described in the Materials and Methods. Panel A: curve a represents nonspecific staining of cells incubated only with biotinylated-8E3 and streptavidin-PE. Curves b and c represent cells incubated with 100 and 500 ng/ml of human IL-12, respectively. Panel B: curve a represents nonspecific staining, and curves b, c, d, and e represent cells incubated with 2.5, 12.5, 125 and 500 ng/ml of rp40, respectively.

Figure 2. Specificity of rp40 binding to KIT225/K6 cells detected by FACS analysis. Purified human IL-12 (A), conditioned media from cultures of COS cells cotransfected with human p35 and p40 cDNAs (B), or with human p40 cDNA alone (C) were diluted to 0.5 μ g/ml (determined by EIA) and incubated with 4A1 neutralizing monoclonal anti-human IL-12 antibody (b) or with normal rat IgG (R-IgG) (c) at a final concentration of 25 μ g/ml at room temperature for 1 h prior to addition of KIT225/K6 cells. Conditioned medium from culture of COS cells transfected with pEF-BOS wild type plasmid was used as a control (D). To measure nonspecific staining, cells were incubated with only biotin-8E3 and streptavidin-PE (a).

Figure 3. Proliferation of PHA-activated human lymphoblasts in response to conditioned media containing individually expressed rp40 and rp35, or coexpressed rp35/rp40. Human PHA(phytohemagglutinin)-blasts were cultured with serial dilutions of the conditioned media from cultures of COS cells transfected with human p35 and p40 cDNA (-●-), p40 cDNA alone (-■-), p35 cDNA alone (-▲-), or pEF-BOS wild type plasmid (-○-). [3 H]thymidine incorporation was measured after 48 h as described in Materials and Methods.

Figure 4. Western blot analysis of COS-expressed human rp35, rp40 and rp35/rp40 heterodimer proteins. Conditioned media (0.5 ml) were immunoprecipitated with 5 μ g IgG protein isolated from goat anti-human IL-12 antisera, separated by SDS/PAGE under nonreducing (A) or reducing (B) conditions and analyzed by immunoblot using rabbit anti-human IL-12 antisera and peroxidase-conjugated donkey anti-rabbit IgG. Samples loaded to each lane were as indicated. Human IL-12 from CHO cells was loaded with two different doses (50 ng and 200 ng, respectively) for comparison. Positions of molecular weight standards ($\times 10^{-3}$) are shown on the left.

Figure 5. Deglycosylation of COS-expressed human rp40 proteins. Purified human IL-12 (0.5 μ g) and COS-expressed human rp40 proteins immunoprecipitated with goat anti-human IL-12 antisera were deglycosylated by N-deglycosidase F as described in Materials and Methods. Duplicate samples of the deglycosylated proteins were separated by SDS/PAGE under nonreducing (A) or reducing (B) conditions and analyzed by immunoblot as described in Materials and Methods. Positions of molecular weight standards ($\times 10^{-3}$) are shown on the left.

Figure 6. HPLC fractionation of rp40 species. Recombinant p40 proteins were partially purified by immunoaffinity chromatography and applied onto a HiLoad Superdex 75 gel filtration column. The fractions were evaluated in the p40 EIA and the KIT225/K6 FACS binding assay. The EIA data (-○-) were

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plotted as $\mu\text{g/ml}$ (using human IL-12 as a standard), and the binding data (-•-) were plotted as the mean peak of fluorescence intensity (top panel). The EIA positive fractions were evaluated by nonreducing SDS-PAGE and W stem blot analysis (bottom panel). Lanes 1 to 12 represent the proteins (~50 ng) from fractions 40, 44, 46, 48, 50, 52, 54, 58, 60, 62, 64, and 70, respectively.

5 Figure 7. Inhibition of [^{125}I]human IL-12 binding to human PHA-blasts by COS-expressed rp40 proteins. Varying concentrations of purified human IL-12 heterodimer (-●), COS-expressed rp40 homodimer (-O-) or rp40 monomer (-■-) (determined by EIA using IL-12 as standard) were incubated with 1×10^6 PHA-blasts in the presence of 100 pM [^{125}I]human IL-12 for 1.5 h at room temperature. The data represent specific binding of [^{125}I]IL-12 and are expressed as percentage of the amount of [^{125}I]IL-12 bound to the cells in the presence of the indicated concentration of unlabeled IL-12 or rp40 proteins when compared with the total specific binding in the absence of unlabeled IL-12.

10 Figure 8. COS-expressed human p40 homodimer induces little proliferation of human PHA-blasts. Serial dilutions of purified native human IL-12 (-O-), partially purified COS-expressed human rp40 homodimer (-●-) or PBS buffer (-□-) were incubated with 2×10^4 PHA-blasts. Proliferation was measured in a 48 h assay as described in Materials and Methods. The concentration of rp40 was determined by a sandwich EIA using native human IL-12 as standard as described in Materials and Methods.

15 Figure 9. Inhibition of IL-12 bioactivity by COS-expressed p40 homodimer. Varying concentrations of COS-expressed human rp40 homodimer were mixed with 0.1 ng/ml of native human IL-12 prior to incubation with 2×10^4 PHA-blasts. Neutralization of IL-12 bioactivity by COS-expressed p40 homodimer was measured in a 48 h proliferation assay as described in Materials and Methods. The data are expressed as the % inhibition of [^3H]thymidine incorporation in the presence of the indicated concentration of p40 homodimer as compared to [^3H]thymidine incorporation in the presence of an equivalent dilution of PBS buffer. The concentration of p40 was determined by a sandwich EIA using native human IL-12 as standard as described in Materials and Methods.

20 Figure 10. Models of IL-12 p35/p40 heterodimer and p40/p40 homodimer binding to the IL-12 receptor and signal transduction. The IL-12 p40 subunit has to be associated with the p35 subunit or with another p40 molecule for proper conformation of the epitopes required for binding to the IL-12 receptor. However, only the heterodimer (A), not the homodimer (B) acts as a full agonist to induce signaling.

The present invention is directed to homodimer proteins of p40 subunits of interleukin-12 capable of 25 binding to the interleukin-12 receptor but being unable to mediate cellular proliferation.

The term "homodimer" comprises the association of two p40 subunits to one another. The association of p40 subunits is of covalent or non-covalent character and may be achieved in vivo, for example by recombinant expression of p40 subunits in suitable host cells by post translational modifications or in vitro, for example by chemical means such as cross-linking agents.

30 The term "p40 subunit" includes the natural and recombinant p40 subunit of interleukin-12 as well as derivatives thereof. The term comprises fragments of the p40 subunit as well as fusion proteins: i.e. p40 subunit derivatives comprising the amino acid sequence of natural p40 or partial sequences thereof together with amino acid sequences derived from other proteins. The protein according to the invention may optionally contain an initiator methionine.

35 The term "p40 subunit" also comprises non-naturally occurring p40 analogous subunits having amino acid sequences which are analogous to the amino acid sequence of p40 or its fragments. Such p40 analogue subunits are proteins in which one or more of the amino acids of the natural p40 or its fragments have been replaced or deleted without loss of the mentioned p40 homodimer activity. Such analogues may be produced by known methods of peptide chemistry or by known methods of recombinant DNA technology such as site directed mutagenesis.

40 Furthermore the terms "p40 homodimer proteins" and "p40 subunits" also include "functional derivatives". This term refers to derivatives of the p40 homodimer protein and to the p40 subunit, which may be prepared from the functional groups occurring as side chains on the residues or the N- or C-terminal groups, by means known in the art, and are included in the invention as long as they remain pharmaceutically acceptable, i.e. they do not destroy the activity of the protein and do not confer toxic properties on compositions containing it. These derivatives may include, for example, polyethylene glycol side-chains which may mask antigenic sites and extend the residence of the p40 homodimer protein in body fluids. Other derivatives include aliphatic esters of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives of free amine groups of the amino acid residues formed with acyl moieties (e.g. alkanoyl or carbocyclic aryl groups) or O-acyl derivatives of free hydroxyl groups (for example that of seryl- or threonyl residues) formed with acyl moieties.

45 A preferred embodiment of the invention is a p40 homodimer protein consisting of two p40 subunits of interleukin-12, preferably associated by at least one disulphide bond. The molecular weight of this

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compound is about 80 kD. The preferred p40 subunit is that of SEQ ID NO:1.

The p40 homodimer proteins of this invention are capable of binding to the interleukin-12 receptor but unable to mediate cellular proliferation: they act as interleukin-12 receptor antagonists. This biological activity can be measured by standard assays known in the art (EP 0 443 827), for example as described below.

In accordance with the present invention, the p40 homodimer protein is obtained in pure form. Based on the sequence of the p40 subunit of interleukin-12 (SEQ ID NO:1), which is obtainable by methods known in the art (EP 0 433 827), biologically active analogues and fragments can be prepared of the p40 subunits and p40 homodimer proteins, respectively. These biologically active proteins may be produced biologically using standard methods of the recombinant DNA technology or may be chemically synthesized in an amino acid synthesizer or by manual synthesis using well-known liquid or solid phase peptide synthesis methods. In a similar way analogues, fragments and proteins comprising the amino acid sequence of p40 together with other amino acids can be produced. All of these proteins may then be tested for the corresponding biological activity.

Thus the present invention relates to p40 homodimer proteins, its use and methods for the preparation thereof.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA and immunology, which are within the skills of an artisan in the field. Such techniques are explained fully in the literature. See e.g., Sambrook, Fritsch & Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL (1989); DNA CLONING, VOLUMES I AND II (D.N. Glover ed., 1985); OLIGONUCLEOTIDE SYNTHESIS (M.J. Gait ed., 1984); NUCLEIC ACID HYBRIDIZATION (B.D. Hames & S.J. Higgins eds., 1984); TRANSCRIPTION AND TRANSLATION (B.D. Hames & S.J. Higgins eds., 1984); ANIMAL CELL CULTURE (R.I. Freshney ed., 1986); IMMOBILIZED CELLS AND ENZYMES (IRL Press, 1986); B. Perbal, A PRACTICAL GUIDE TO MOLECULAR CLONING (1984); the series, METHODS IN ENZYMOLOGY (Academic Press, Inc.); GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (J.H. Miller and M.P. Calos eds., 1987, Cold Spring Harbor Laboratory), Methods in Enzymology Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively); IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY (Mayer and Walker, eds., 1987, Academic Press, London), Scopes, PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, second Edition (1987, Springer-Verlag, N.Y.), and HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, VOLUMES I-IV (D.M. Weir and C.C. Blackwell eds., 1986).

The DNA sequences and DNA molecules encoding a p40 subunit of the present invention may be expressed using a wide variety of host/vector combinations. For example, useful vectors may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Examples of such vectors are viral vectors, such as the various known derivatives of SV40, bacterial vectors, such as plasmids from *E. coli* including pCR1, pBR322, pMB9 and RP4, phage DNAs, such as the numerous derivatives of phage λ, M13 and other filamentous single-stranded DNA phages, as well as vectors useful in yeasts, such as the 2 μ plasmid, vectors useful in eucaryotic cells more preferably vectors useful in animal cells, such as those containing SV40, adenovirus and/or retrovirus derived DNA sequences. Useful vectors may be also derived from combinations of plasmids and phage DNA's, such as plasmids which have been modified to comprise phage DNA or other derivatives thereof.

Expression vectors which may be used for the preparation of recombinant p40 homodimer proteins are characterized by comprising at least one expression control sequence which is operably linked to the p40 DNA sequence inserted in the vector in order to control and to regulate the expression of the cloned p40 DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage λ, the control region of fd coat protein, the glycolytic promoters of yeast, e.g., the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, e.g., Pho 5, the promoters of the yeast α-mating factors, and promoters derived from polyoma virus, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters of SV40, and other sequences known to control the expression of genes of prokaryotic or eucaryotic cells and of their viruses as well as combinations of the said promoter/operator sequences.

The DNA coding for the p40 subunit is known (2,4-6,13). The DNA may be obtained by conventional cloning techniques or by polymerase chain reaction (PCR) using the primers complementary to the beginning and end of the p40 subunit cDNA coding sequences (6, 13).

The present invention also provides host cells and expression vectors for the preparation of p40 homodimer. The method comprises culturing a suitable cell or cell line, which has been transformed with a DNA sequence coding for expression for a p40 monomer under control of known regulatory sequences. Suitable cells or cell lines may be eucaryotic cells, such as COS cells, SF9 cells for the Baculovirus

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expression system or prokaryotic cells, such as *E. coli*. The selection of further host cells, expression vectors and methods for transformation, culture, amplification, screening and product production are known in the art (17). Suitable expression vectors are pEF-BOS (16a) for COS-expression and pACDZ-1 for Baculovirus expression system (16c).

6 The present invention also provides methods for recovering of the p40 homodimer protein, e.g. via immunoaffinity, gel filtration chromatography and gel electrophoresis. Further, the p40 homodimer produced by fermentation of the prokaryotic and eucaryotic hosts transformed with the DNA sequences of this invention can then be purified to essential homogeneity by known methods such as, for example, by centrifugation at different velocities, by precipitation with ammonium sulphate, by dialysis (at normal 10 pressure or at reduced pressure), by preparative isoelectric focusing, by preparative gel electrophoresis or by various chromatographic methods such as gel filtration, high performance liquid chromatography (HPLC), ion exchange chromatography, reverse phase chromatography and affinity chromatography (e.g. on Sepharose™ Blue CL-6B or on carrier-bound monoclonal antibodies directed to the IL-12 homodimer).

15 Medicaments containing the IL-12 homodimer are also an object of the present invention as is a process for the manufacture of such medicaments, which process comprises bringing the IL-12 homodimer and, if desired, one or more other therapeutically valuable substances into a galenical administration form.

20 p40 homodimer protein or the corresponding pharmaceutical compositions may be administered orally, for example in the form of tablets, coated tablets, dragées, hard or soft gelatine capsules, solutions, emulsions or suspensions. Administration can also be carried out rectally, for example using suppositories; locally or percutaneously, for example using ointments, crèmes, gels or solutions; or parenterally by injection or by gradual perfusion over time. It can be administered intravenously, intraperitoneally, intramuscularly or subcutaneously.

25 For the preparation of tablets, coated tablets, dragées or hard gelatine capsules the compounds of the present invention may be admixed with pharmaceutically inert, inorganic or organic excipients. Examples of suitable excipients for tablets, dragées or hard gelatine capsules include lactose, maize starch or derivatives thereof, talc or stearic acid or salts thereof.

30 Suitable excipients for use with soft gelatine capsules include for example vegetable oils, waxes, fats, semi-solid or liquid polyols etc.; according to the nature of the active ingredients it may however be the case, that no excipient is needed at all for soft gelatine capsules.

35 For the preparation of solutions and syrups, excipients which may be used include for example water, polyols, saccharose, invert sugar and glucose.

40 Pharmaceutically acceptable carriers and preparations for parenteral administration include sterile or aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, anti-microbials, anti-oxidants, chelating agents, inert gases and the like. See, generally, *Remington's Pharmaceutical Science*. 18th Ed., Mack Eds., 1990.

45 For suppositories, and local or percutaneous application, excipients which may be used include for example natural or hardened oils, waxes, fats and semi-solid or liquid polyols.

The pharmaceutical compositions may also contain preserving agents, solubilising agents, stabilising agents, wetting agents, emulsifiers, sweeteners, colorants, odorants, salts for the variation of osmotic pressure, buffers, coating agents or antioxidants. They may also contain other therapeutically valuable agents.

50 The p40 homodimer to be administered to a human to get a biological response should be given preferably intramuscularly or intravenously 2 to 3 times per week. The expected dose range is 0.1 to 2 mg/kg of body weight, although the dose ranges of the p40 homodimer may be determined by those of ordinary skill in the art without undue experimentation.

The invention also relates to method for preparing a medicament or pharmaceutical composition comprising the p40 homodimer protein of the invention.

55 The IL-12 p40 homodimer is useful as an IL-12 antagonist to block the biological activity of IL-12 in pathologic immune responses. Current evidence from both *in vitro* and *in vivo* studies suggest that IL-12 plays an important role in the development of Th1-type helper T cells which promote cell-mediated immun responses (22, 24), in triggering gamma Interferon production by mature T and/or NK cells (25), and in facilitating specific cytolytic T lymphocyte responses (26). Excessive activity of Th1 cells (27, 28) and/or

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excessive production of gamma interferon (27-31) may be involved in the pathogenesis of some autoimmune disorders and septic shock, indicating that IL-12 p40 homodimer is useful in the treatment of disorders such as rheumatoid and other inflammatory arthritides, Type I diabetes mellitus, multiple sclerosis, systemic lupus erythematosus, septic shock, etc. In addition, IL-12 p40 homodimer is useful in preventing or delaying homograft rejection and graft versus host disease. In using IL-12 p40 homodimer to prevent or reverse pathologic immune responses, it can be combined with other cytokine antagonists such as antibodies to the IL-2 receptor, soluble TNF receptor, or the IL-1 receptor antagonist, and the like.

MATERIALS AND METHODS

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Cell lines.

KIT225/K6, an IL-2-dependent subclone derived from the human T cell line KIT225 (14) were previously found to express IL-12 receptors (15). KIT225/K6 cells were cultured in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 2 mM L-glutamine (Sigma, St. Louis, MO), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Grand Island, NY), 15% FCS (JRH Biosciences, Lenexa, KS), and 100 U/ml human rIL-2 (Hoffmann-La Roche Nutley, NJ). COS (ATCC CRL 1650 or 1651) cells were cultured in DMEM (Gibco) with 4500 mg/liter glucose, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 10% FCS (JRH Biosciences).

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Expression of IL-12 subunits.

The IL-12 expression constructs for COS-expression were built in the pEF-BOS vector which contains the promoter of the human polypeptide chain elongation factor 1 α (EF-1 α) chromosomal gene (16a). The cDNA fragments containing the entire coding region of the human or mouse p40 or p35 cDNAs generated by polymerase chain reaction (PCR) using the primers complementary to the beginning and end of the subunit cDNA coding sequences as described (6, 13) were subcloned individually into the pEF-BOS vector at the Xba I cloning site by blunt end ligation (17). The ligation products were transformed into E. coli strain DH-5 alpha (BRL-Gibco), and the resulting colonies were screened by PCR for the correct insert orientation by using a forward primer within the pEF-BOS promoter and a reverse primer within the subunit coding sequences. Positive clones were selected and amplified in a suitable E. coli strain, for example MC1161. Plasmid DNAs were prepared by using the QIAGEN plasmid kit (Qiagen, Chatsworth, CA) and transfected into COS cells by using the DEAE dextran/chloroquine method (17). The DNAs at a concentration of 2 µg/ml were mixed with 10% Nutridoma-SP (Boehringer Mannheim, Indianapolis, IN), 0.5 mg/ml DEAE dextran and 0.05 mg/ml chloroquine in DMEM (Dulbecco's modified essential medium) medium and applied to COS cells seeded for 16 h. After a 2.5-3 h incubation, the cells were treated with 10% DMSO in serum free DMEM medium for 3 min followed by washing with DMEM medium, and then cultured in DMEM/10% FCS medium. Supernatant fluids were collected from the cultures of transfected COS cells after 72 h. Coexpression of p40 and p35 subunits was performed by mixing the two plasmid DNAs at a 1:1 (W/W) ratio in transfection reagents. The supernatant fluids derived from the COS cultures transfected with pEF-BOS wild-type plasmid DNA were used as controls.

The human IL-12 p40 construct for expression in Baculovirus system was built in pACDZ-1 vector (16b,16c) at BamH1 site by using same approach described above. A recombinant baculovirus expressing the p40 chain was generated by cotransfected SF9 cells (ATCC CRL 1711) with wild type baculovirus DNA and the p40 expressing plasmid pACDZ-1. Limited dilution cloning in microtiterplates was used to isolate a single recombinant baculovirus expressing the human IL-12 p40 subunit.

IL-12 receptor binding and proliferation assays.

50 The binding of COS-expressed IL-12 molecules to IL-12 receptor-bearing cells was measured by FACS (fluorescence activated cell sorting) analysis essentially as described by Desai et al.(10). Briefly, 1 x 10⁶ KIT225/K6 cells suspended in 25 µl of FACS buffer (PBS (phosphate-buffered-saline)/2% FCS/0.05% sodium azide) were incubated with IL-12 preparations (25 µl) at room temperature for 40 min, followed by incubation with biotinylated mAb 8E3, a non-inhibitory anti-human IL-12, p40 specific monoclonal antibody, (5 µg/ml, 50 µl), (11) for 30 min, and then with streptavidin-PE (1.5 µg/ml, 50 µl; FisherBiotech, Pittsburgh, PA) for 20 min. The stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson). Specificity of binding was determined by pre incubating the IL-12 preparations (0.5 µg/ml) with 4A1 (25 µg/ml), a rat inhibitory anti-human IL-12 monoclonal antibody, prior to adding cells. Control samples were

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incubated with normal rat IgG (25 µg/ml). The receptor binding properties of the COS-expressed IL-12 molecules were also evaluated in an [¹²⁵I]IL-12 competitive receptor binding assay performed essentially as previously described (11). 0.1 ml aliquots of serial dilutions of culture supernatant fluids or purified IL-12 were mixed with 0.05 ml aliquots of binding buffer (RPMI-1640, 5% FCS, 25 mM HEPES pH 7.4) containing [¹²⁵I]IL-12 (2x10⁶ cpm). The mixture was added to 0.1 ml of activated blasts (1x10⁷ cell/ml) and incubated in a shaking water bath at 25 °C for 1.5 h. Non-specific binding was determined by inclusion of 20 µg/ml unlabeled IL-12 in the assay. Incubations were carried out in duplicate. Cell bound radioactivity was separated from free [¹²⁵I]IL-12 by centrifugation of 0.1 ml aliquots of the assay contents in duplicate through 0.1 ml silicone oil for 90 sec at 10,000 x g. The tip containing the cell pellet was excised and cell bound radioactivity was determined in a gamma counter.

The biological activity of COS-expressed IL-12 molecules was evaluated in proliferation assays using 4-day PHA-activated human lymphoblasts previously described (4, 13).

Anti-IL-12 antibodies and sandwich enzymatic immunoassay (EIA).

Goat and rabbit anti-human IL-12 antisera were obtained from animals immunized with purified human rIL-12 that had been expressed in CHO cells (35). The IgG fraction was isolated from 100 ml of the antisera by Protein-G Sepharose (Pharmacia LKB, Piscataway, NJ) affinity chromatography according to the manufacturer's procedures. Anti-human IL-12 antibodies were purified from the IgG fractions on a human IL-

12-conjugated hydrazide AvidGel F (BioProbe International) immunoaffinity column (1.5 X 2.0 cm, 0.55 mg protein per ml resin). Biotinylation of the antibodies using Biotin X-NHS (Calbiochem, San Diego, CA) was performed as described (18). Monoclonal antibodies 4A1 and 8E3 are rat antibodies specific for the p40 subunit of human IL-12 (EP 0 433 827, 11).

The IL-12 sandwich EIA, using mAb 4A1 as a capture antibody and peroxidase-conjugated 8E3 as detection antibody, was performed as described previously (11). This assay detects IL-12 heterodimer and p40 subunit but not p35 subunit. Therefore, a second IL-12 sandwich EIA using polyclonal antibodies was developed to detect both p40 and p35. In this assay, 96 well EIA plates (Nunc MaxiSorp, Thousand Oaks, CA) were coated with affinity-purified goat anti-human IL-12 antibody (2 µg/ml, 50 µl/well) at 4 °C overnight and blocked with 1% BSA in PBS pH 7.4 for 1 h at RT. Serial dilutions of IL-12 and culture supernatant fluids were applied to the plates, and incubated at RT for 2.5 h. The plates were subsequently incubated with biotinylated, affinity-purified rabbit anti-human IL-12 antibody (500 ng/ml, 50 µl/well), followed by peroxidase-conjugated streptavidin (1 µg/ml, 50 µl/well, Sigma, St. Louis, MO). Color was developed with 100 µl of 1 mM ABTS (2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)/0.1% (v/v) H₂O₂, and the absorbance at 405 nm was determined with a Vmax Kinetic Microplate reader (Molecular Devices, Palo Alto, CA).

All values are based on an IL-12 standard curve with no corrections calculated for differences in molecular weights of monomers or dimers.

Immunoprecipitation.

Immunoprecipitation of COS-expressed IL-12 subunits and heterodimers was performed as described (17). Briefly, 0.5 ml supernatant fluids from transfected COS cultures were incubated with 5 µg IgG protein isolated from goat anti-IL-12 antiserum at 4 °C on a rotating mixer overnight. The immune complexes were adsorbed onto Protein G-Sepharose (50% suspension, 10 µl, Pharmacia LKB) at 4 °C for 2 h, and the beads were washed twice with 1 ml NET-Gel buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Nonidet P-40, 1 mM EDTA, 0.25% (w/v) gelatin and 0.02% (w/v) sodium azide), and once with 1 ml of 10 mM Tris-HCl (pH 7.5) containing 0.1% (v/v) Nonidet P-40. The bound proteins were dissociated from the beads by heating for 3 min at 95 °C in either reducing (10% 2-ME) or non-reducing SDS sample buffer.

SDS-PAGE and Western blotting.

SDS-PAGE was performed according to the method of Laemmli (19). Western blotting was performed by electrophoretically transferring proteins to a nitrocellulose membrane (0.2 µ) (MSI, Westboro, MA). The transferred membranes were blocked by incubation in PBST buffer (PBS with 0.05% v/v Tween-20) containing 5% (w/v) non-fat dry milk, and then probed with anti-IL-12 rabbit antisera (1:500 dilution). After three washes with PBST buffer, the membranes were incubated at room temperature with peroxidase-conjugated donkey anti-rabbit IgG antibodies (1:1000 dilution) (Jackson Immuno Research, West Grove, PA). The color was developed by use of 4-chloro-1-naphthol (BioRad, Richmond, CA) in 20 mM Tris-HCl buffer (pH 7.5) containing 0.1% (v/v) H₂O₂.

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Purification of COS-expressed p40.

One liter of conditioned media containing approximately 3 µg/ml of human recombinant p40 (rp40) was applied to a mAb 4A1-conjugated NuGel (NHS) immunoaffinity column (2.5 x 10 cm, containing 1.6 mg antibody per ml gel) (35) at a flow rate of 2 ml/min, and the column was washed extensively with PBS containing 0.5 M NaCl and 0.2% Tween 20 until absorbance monitoring at 280 nm was less than 0.01. The bound proteins were then eluted with 100 mM glycine/150 mM NaCl (pH 2.8) at a flow rate of 2 ml/min, and 20 ml fractions were collected and immediately neutralized with 1/10 vol. of 1 M Tris-HCl (pH 8.0). The ELA-positive fractions were pooled, dialyzed against PBS overnight at 4 °C, concentrated by ultrafiltration using YM 10 membranes (Amicon, Beverly, MA) to 5 ml, and applied onto a HiLoad Superdex 75 (Pharmacia LKB) column (1.6 x 60 cm) equilibrated with Dulbecco's PBS buffer. The column was eluted at a flow rate of 1 ml/min with the same buffer, and 1 ml fractions were collected. Proteins from each fraction were examined by EIA, SDS-PAGE and Western blot analysis.

15 Deglycosylation.

500 ng of pure human IL-12 or immunoprecipitated rp40 protein was denatured by heating at 95 °C for 5 min in 0.25 M Na₂HPO₄ (pH 7.2), 0.5% SDS with or without 1% 2-ME. The samples were cooled to room temperature, adjusted to 1% Nonidet P-40, 20 mM EDTA, and then treated with 0.1 U of N-glycosidase F (Boehringer Mannheim, Indianapolis, IN) at 37 °C for 24 h. The deglycosylated proteins were examined by SDS-PAGE and Western blot analysis.

Amino-terminal sequence analysis of COS-expressed p40.

25 The immunoaffinity purified r40 proteins were separated on 10% non-reducing SDS gel and transferred electrophoretically to an Immobilon™ PVDF membrane (Millipore, Bedford, MA). The bands at ~80 and ~40 kDa identified by Coomassie blue staining were subjected to automated Edman degradation on an Applied Biosystems Model 470A gas-phase sequencer with on-line analysis of phenylthiohydantoin (PTH) amino acid derivatives as described previously (20).

30

TABLE I

Amino-terminal Sequences of COS-expressed Human p40 Monomer, p80 Homodimer and Native Human IL-12 p40 Subunit		
35	Protein	Sequence
	Native Human p40	IWEKKDVYV ^a (SEQ ID NO:2)
	rp40 Dimer	Iw ^b ELkkDVYV (SEQ ID NO:2)
40	rp40 Monomer (band 1)	IwELkkDVYV (SEQ ID NO:2)
	rp40 Monomer (band 2)	IWEELkkDVYV (SEQ ID NO:2)

a. From Podlaski et al., 1991

b. Small case letter represents a signal with a recovery less than 2 pmol.

45

Expression and characterization of human IL-12 subunits.

Human IL-12 subunits p35 and p40, or human IL-12 p35/p40 heterodimer were expressed by transfecting either subunit cDNA independently or cotransfeting both cDNAs at a 1:1 (w:w) ratio in COS cells. Secretion of the recombinant proteins was evaluated by two different EIA's. The p40-specific monoclonal antibody-based EIA was capable of detecting the p40 subunit and the p40/p35 heterodimer. The IL-12-specific polyclonal EIA was also capable of detecting the p35 subunit. Using human IL-12 as a standard, the concentration range of rp40 and rp35/rp40 proteins in the conditioned media was 0.5-3.0 µg/ml, whereas the expression of rp35 alone was approximately 0.2 µg/ml. It remains unclear whether the p35 expression was low or the sensitivity of the polyclonal EIA in detecting p35 was poor.

The COS-expressed human IL-12 recombinant proteins were initially examined for their ability to inhibit the binding of [¹²⁵I]human IL-12 to PHA-activated human lymphoblasts. The rp40 supernatants at a 1:2 dilution exhibited 30-40% inhibition of [¹²⁵I]IL-12 binding in three independent experiments, whereas the

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rp35 supernatants were inactive. The binding of rp40 to the IL-12 receptor was further characterized by flow cytometry using KIT225/K6 cells which constitutively express IL-12 receptors (IL-12R) (15). Dose-dependent binding of human IL-12 and rp40 to KIT225/K6 was observed in the range of 2.5-500 ng/ml (Fig. 1). Specificity of the binding was demonstrated by achieving greater than 80% inhibition of the binding by

5 preincubation of IL-12 or rp40 with an inhibitory rat anti-human p40 monoclonal antibody, 4A1 (Fig. 2). Normal rat IgG had no effect on IL-12 or rp40 binding.

Conditioned media containing the COS-expressed IL-12 subunit proteins were evaluated in the human PHA-blast proliferation assay (Fig. 3). The rp35/rp40-containing medium supported T cell proliferation in a dose-dependent manner with an apparent EC_{50} of 8 ng/ml. The rp40 supernatants did not induce proliferation at concentrations equivalent to the rp35/rp40 supernatant.

Characterization of the rp40 40 kD and 80 kD species.

The recombinant human IL-12 subunits were immuno-precipitated with anti-human IL-12 goat antiserum and characterized by SDS-PAGE and Western blot analysis. Analysis of the rp40 expressed by COS cells transfected with only the p40 cDNA revealed two sets of multiple bands under nonreducing conditions with heterogeneous molecular weights of 70-85 kD and 35-45 kD (Fig. 4A). Under reducing conditions, only three closely spaced bands at approximately 38-49 kDa were identified suggesting that the 80 kD proteins are disulfide-linked rp40 homodimers (Fig. 4B). Treatment of the rp40 immunoprecipitates with N-de-glycosidase-F shifted both molecular weight species down to smaller products under nonreducing conditions (Fig. 5A), and converted the reduced triple bands to a single 38 kDa product similar to p40 subunit of the deglycosylated human IL-12 (12) demonstrating that the multiple bands of rp40 expressed in COS cells are due to glycosylation heterogeneity.

In contrast, the immunoprecipitation of rp35 protein revealed only a single band with a molecular weight of 35 kD under reducing conditions (Fig. 4B). Under nonreducing conditions, a set of lightly stained bands were found at 60-70 kD suggesting that rp35 may also partially form dimers. However, the polyclonal goat anti-IL-12 antibody poorly recognized the rp35 proteins (Fig. 4A). Coexpression of p35 and p40 gave a pattern of bands which was a mixture of those seen when each subunit was expressed independently (Fig. 4).

30 To confirm the identity of the two rp40 species, the rp40 proteins were partially purified by 4A1 immunoaffinity chromatography. Only 60% of EIA positive material was recovered by elution with 100 mM glycine containing 150 mM NaCl at pH 2.8. The 4A1 affinity-purified proteins were then separated by SDS-PAGE, electrophoretically transferred to a PVDF membrane, and subjected to amino acid microsequencing. One broad band at ~80 kD and two bands at 35-40 kD gave NH₂-terminal sequences identical to that of native human IL-12 p40 purified from NC-37 cells (4, 12) (Table I). No trace of p35 sequences as identified with the rp40 species. This result confirmed that the 80 kD protein is a p40 homodimer.

The immunoaffinity purified p40 proteins were further fractionated by Superdex-75 gel filtration chromatography. Two EIA positive protein peaks were identified at molecular weights corresponding to 80 kD and 40 kD (Fig. 6A). SDS-PAGE and Western blot analysis of the fractions confirmed the separation of dimer from monomer rp40 (Fig. 6B). The ratio of the monomer to dimer varied from experiment to experiment, but, on the average, approximately 30% of the COS-expressed rp40 was p40 homodimer.

The Superdex 75 column fractions were tested for binding to KIT225 cells by FACS analysis. Binding activity correlated only with the 80 kDa p40-EIA positive protein (Fig. 6). The 80 and 40 kD peak fractions were pooled separately, concentrated and examined in the competitive radioligand receptor binding assay (Fig. 7). The 80 kD protein pool inhibited [¹²⁵I]human IL-12 binding to PHA-blasts with an IC_{50} of 80 ng/ml, which is similar to the IC_{50} of human IL-12 heterodimer (20 ng/ml). However, the slope of the competition curve by the 80 kD homodimer differed from that of IL-12 heterodimer suggesting a different binding interaction with the receptor. The 40 kD protein pool inhibited [¹²⁵I]human IL-12 binding with an IC_{50} about one hundred times higher, which was probably due to a small amount of contamination with the p40 homodimer (Fig. 6B).

The abilities of the rp40 monomer and dimer to support PHA-blast proliferation were also examined (Fig. 8). No proliferative response was observed with either rp40 species even at concentrations 10,000 times higher than that of human IL-12 required to elicit a 50% maximum response. The rp40 dimer was tested for its ability to neutralize IL-12-dependent proliferation of PHA-blasts. The 80 kD protein at varying concentrations was mixed with 0.1 ng/ml of human IL-12 and added to PHA-blasts. Dose-dependent inhibition of IL-12-induced proliferation of PHA-blasts was achieved with an IC_{50} of 1 μ g/ml (Fig. 9).

To clarify the functional role of the individual subunits and localize the epitopes mediating biological and binding activities, the individual subunits were expressed alone or in combination with each other in COS

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cells were expressed and tested the expressed proteins in binding assays and bioassays and by Western blot analysis tested. The rp35 protein was inactive at concentrations as high as 100 ng/ml in the binding and bioassays; however, the rp40 protein reproducibly exhibited binding activity without bioactivity. Analysis of the conditioned media from cultures of COS cells transfected with only the p40 cDNA revealed that such media contained both monomeric p40 and an 80 kD molecule reactive with anti-p40 antibodies. Partial purification of the rp40 by immunoaffinity chromatography and HPLC gel permeation chromatography revealed that the 80 kD protein, but not the 40 kD protein bound to the IL-12R.

The possibility that the 80 kD protein was not a homodimer of p40 but a heterodimer consisting of one IL-12 p40 subunit and a second 35-40 kD exogenous COS-derived protein was investigated. In particular, reports that many cell lines constitutively express IL-12 p35 mRNA (21) raised the possibility that the 80 kD protein could be human IL-12 p40 associated with COS-derived IL-12 p35. Western blot analysis by using p35 specific antibody and deglycosylation experiments (Fig. 5) supported the notion that the 80 kD protein could be reduced to a p40 monomer. The lack of bioactivity despite good binding activity further suggested that the second protein was not a COS-derived p35 IL-12 subunit (assuming no species restriction in the activity of monkey IL-12 on human cells). Also, expression of p40 in a baculovirus system yielded a biologically inactive 80 kD form of p40 capable of binding to the receptor. It seems unlikely that insect cells produce an IL-12-like p35 protein. Most importantly, confirmation of the identity of the 80 kD protein as p40 homodimer was provided by amino acid microsequencing of the protein demonstrating a single N-terminal sequence corresponding to the IL-12 p40 subunit.

In competitive binding analysis, the p40 homodimer was found to bind to the IL-12R nearly as strongly as heterodimeric IL-12, suggesting that the key binding epitopes of IL-12 are localized in the p40 subunit. Though the IC₅₀ values for the heterodimer and the homodimer were similar, 20 and 80 ng/ml respectively, the slopes of the competition curves were different. This suggests a difference in the interaction of the two ligands with the receptor. It is most likely that the p40 binding epitopes are conformational and induced by association with a p35 or a second p40 subunit.

The IL-12 p40 subunit has been previously reported to be produced in excess of heterodimeric IL-12 both by activated B lymphoblastoid lines and by human PBMC stimulated to produce IL-12 (12, 23). It is possible that the p40 homodimer is formed in cells expressing p40/p35 heterodimers.

Based on observations on the roles of the IL-12 subunits in binding and signaling, a model of IL-12 binding to its receptor is illustrated in Figure 10. The p40 subunit contains the receptor binding epitopes that, however, are active only when p40 associates with a second protein, i.e. p35 or another molecule of p40. Both dimeric molecules bind to the IL-12R specifically, but only the dimer containing p35 acts as an agonist to mediate cellular transduction signals (Fig. 10A). In contrast, the p40/p40 dimer behaves as an antagonist to suppress IL-12 mediated responses (Fig. 10B).

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(i) APPLICANT:

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- (A) NAME: F. HOFFMANN-LA ROCHE AG
- (B) STREET: Grenzacherstrasse 124
- (C) CITY: Basle
- (D) STATE: BS
- (E) COUNTRY: Switzerland
- (F) POSTAL CODE (ZIP): CH-4002
- (G) TELEPHONE: 061 - 688 51 08
- (H) TELEFAX: 061 - 688 13 95
- (I) TELEX: 962292/965542 hlr ch

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(ii) TITLE OF INVENTION: P40 HOMODIMER OF INTERLEUKIN-12

(iii) NUMBER OF SEQUENCES: 2

20

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: Apple Macintosh
- (C) OPERATING SYSTEM: System 7.1 (Mac)
- (D) SOFTWARE: Word 5.0

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(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/087,832
- (B) FILING DATE: 02-JUL-1993

(2) INFORMATION FOR SEQ ID NO:1:

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 306 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Ile	Trp	Glu	Leu	Lys	Lys	Asp	Val	Tyr	Val	Val	Glu	Leu	Asp	Trp	Tyr
1														15	

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Pro	Asp	Ala	Pro	Gly	Glu	Met	Val	Val	Leu	Thr	Cys	Asp	Thr	Pro	Glu
													20	25	30

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Glu	Asp	Gly	Ile	Thr	Trp	Thr	Leu	Asp	Gln	Ser	Ser	Glu	Val	Leu	Gly
35												40		45	

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Ser	Gly	Lys	Thr	Leu	Thr	Ile	Gln	Val	Lys	Glu	Phe	Gly	Asp	Ala	Gly
													50	55	60

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Gln Tyr Thr Cys His Lys Gly Gly Glu Val Leu Ser His Ser Leu Leu
 65 70 75 80

5 Leu Leu His Lys Lys Glu Asp Gly Ile Trp Ser Thr Asp Ile Leu Lys
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10 Asp Gln Lys Glu Pro Lys Asn Lys Thr Phe Leu Arg Cys Glu Ala Lys
 100 105 110

15 Asn Tyr Ser Gly Arg Phe Thr Cys Trp Trp Leu Thr Thr Ile Ser Thr
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35 Cys Pro Ala Ala Glu Glu Ser Leu Pro Ile Glu Val Met Val Asp Ala
 180 185 190

40 Val His Lys Leu Lys Tyr Glu Asn Tyr Thr Ser Ser Phe Phe Ile Arg
 195 200 205

45 Asp Ile Ile Lys Pro Asp Pro Pro Lys Asn Leu Gln Leu Lys Pro Leu
 210 215 220

50 Lys Asn Ser Arg Gln Val Glu Val Ser Trp Glu Tyr Pro Asp Thr Trp
 225 230 235 240

Ser Thr Pro His Ser Tyr Phe Ser Leu Thr Phe Cys Val Gln Val Gln
 245 250 255

55 Gly Lys Ser Lys Arg Glu Lys Lys Asp Arg Val Phe Thr Asp Lys Thr
 260 265 270

Ser Ala Thr Val Ile Cys Arg Lys Asn Ala Ser Ile Ser Val Arg Ala
 275 280 285

Gln Asp Arg Tyr Tyr Ser Ser Trp Ser Glu Trp Ala Ser Val Pro
 290 295 300

55 Cys Ser
 305

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ile	Trp	Glu	Leu	Lys	Lys	Asp	Val	Tyr	Val
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Claims

- 20 1. A p40 homodimer of interleukin-12.
2. A p40 homodimer of claim 1 capable of binding to the interleukin-12 receptor without mediating cellular proliferation.
- 25 3. A p40 homodimer of claim 1 or 2 having a molecular weight of about 80 kD.
4. A p40 homodimer of claim 3, wherein the two p40 subunits are associated together by at least one disulfide bond.
- 30 5. A p40 homodimer of claim 4, wherein the p40 subunit is SEQ ID NO:1.
6. A pharmaceutical composition which comprises a pharmaceutically effective amount of a p40 homodimer of interleukin-12 as claimed in any one of claims 1-5, and a pharmaceutically acceptable carrier.
- 35 7. The pharmaceutical composition of claim 6 containing one or more other cytokine antagonists.
8. Use of the p40 homodimer as claimed in any one of claims 1 to 5 for the preparation of a pharmaceutical composition for blocking the biological activity of interleukin-12.
- 40 9. Use of the p40 homodimer as claimed in any one of claims 1 to 5 for the preparation of a pharmaceutical composition for prophylaxis and treatment of pathologic immune responses and septic shock.
- 45 10. Use of the p40 homodimer as claimed in any one of claims 1 to 5 for the preparation of a pharmaceutical composition for treatment of inflammatory arthritis, Type I diabetes mellitus, multiple sclerosis and systemic lupus erythematosus.
11. Use of the p40 homodimer as claimed in any one of claims 1 to 5 for the preparation of a pharmaceutical composition for preventing and/or delaying homograft reaction and graft versus host disease.
- 50 12. Process for producing a p40 homodimer as claimed in any one of claims 1 to 5 characterized in that
 - a. a cell is transformed with an expression vector comprising a cloned gene coding for a p40 subunit of interleukin-12,
 - 55 b. expression of the receptor protein in the transformed cell and
 - c. recovering the p40 homodimer and, if desired, converting it into a functional derivative thereof.



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13. The process of claim 12 wherein the cells are eucaryotic or procaryotic cells, preferably SF9 or COS cells.
14. The process of claim 12 or 13 wherein the expression vector is derived of pEF-BOS or pACD7-1.
- 5 15. Process according to claim 12 to 14 wherein the p40 homodimer of interleukin-12 is recovered by immunoaffinity and gel filtration chromatography.
- 10 16. A p40 homodimer as claimed in any one of claims 1 to 5 prepared by a process as claimed in any one of claims 12-15.
17. A p40 homodimer as claimed in any one of claims 1 to 5 as interleukin-12 receptor antagonist.

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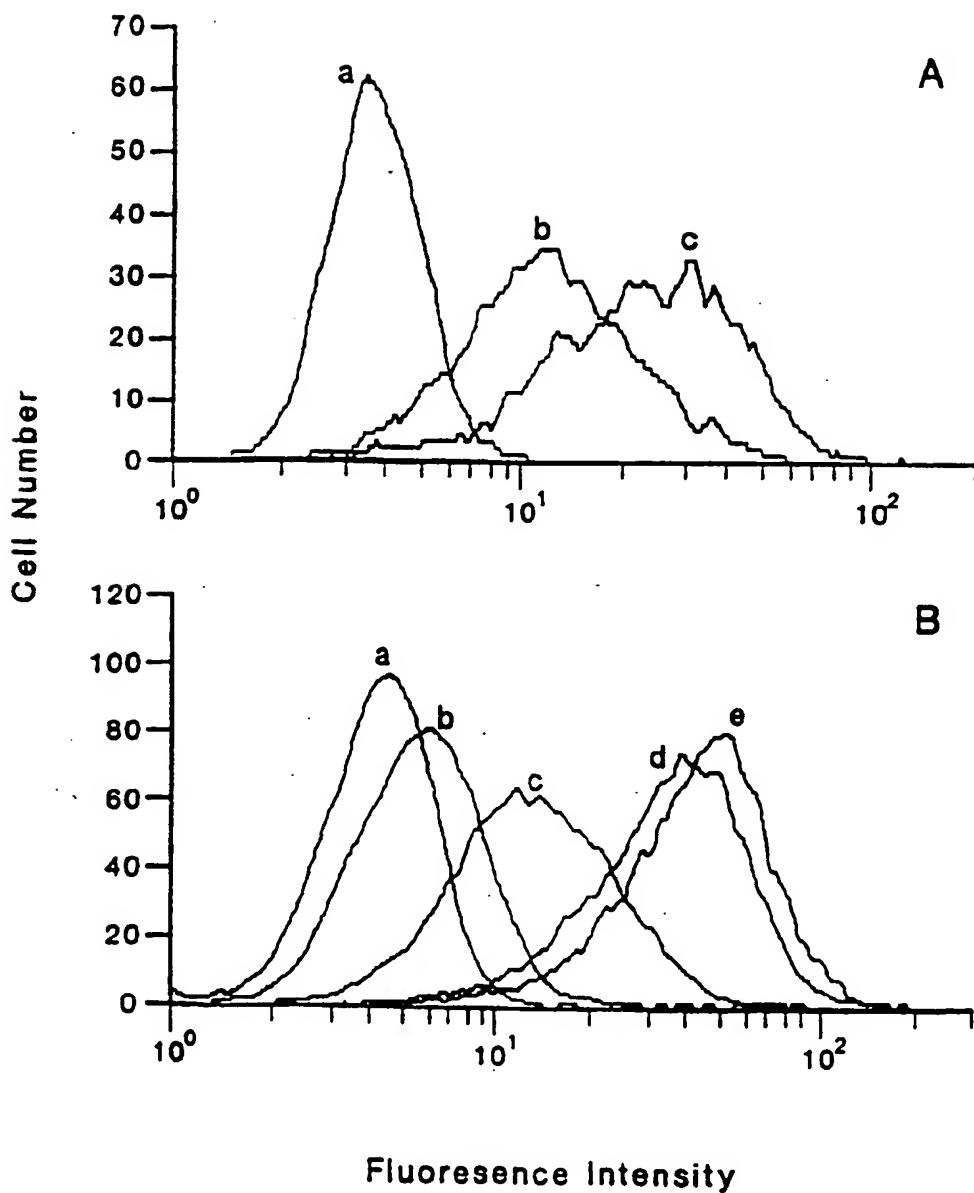
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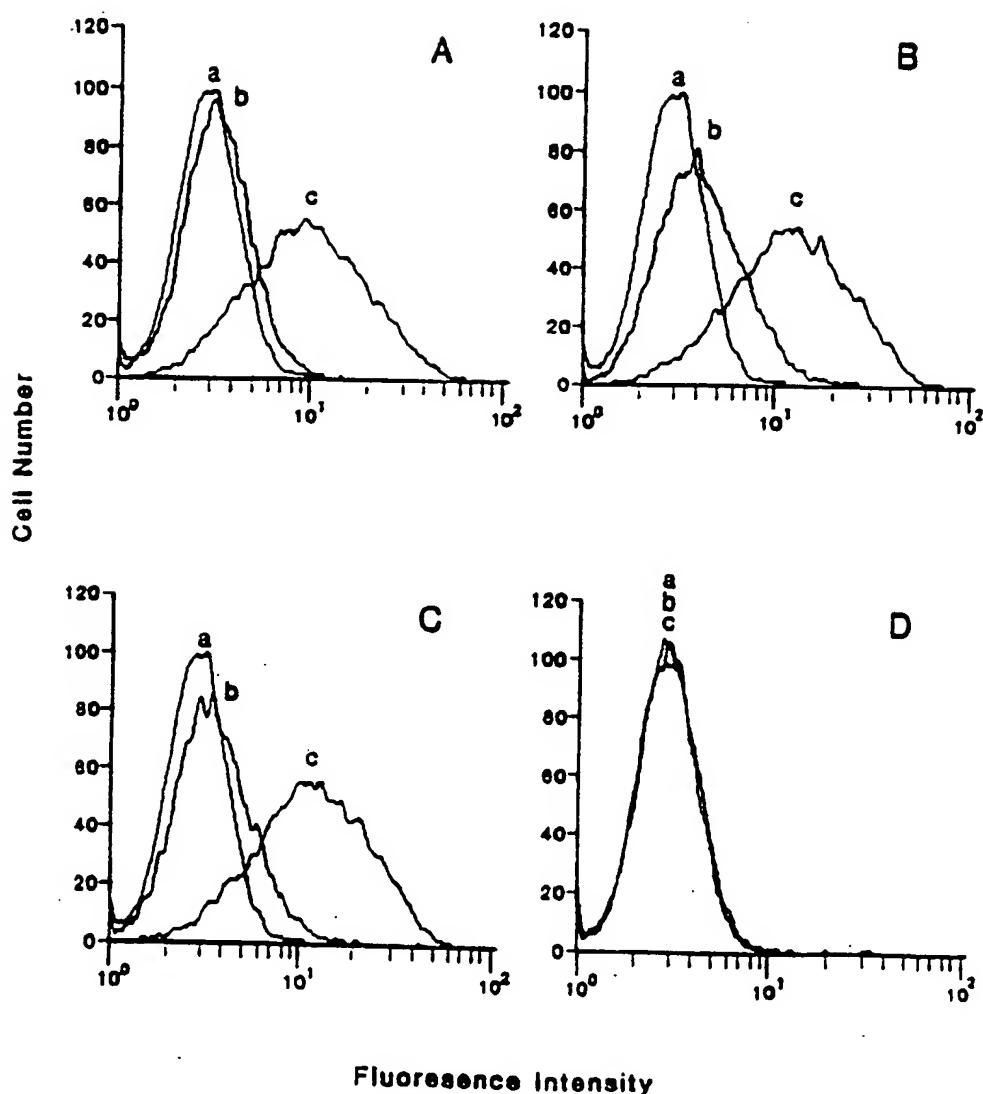
Fig. 1



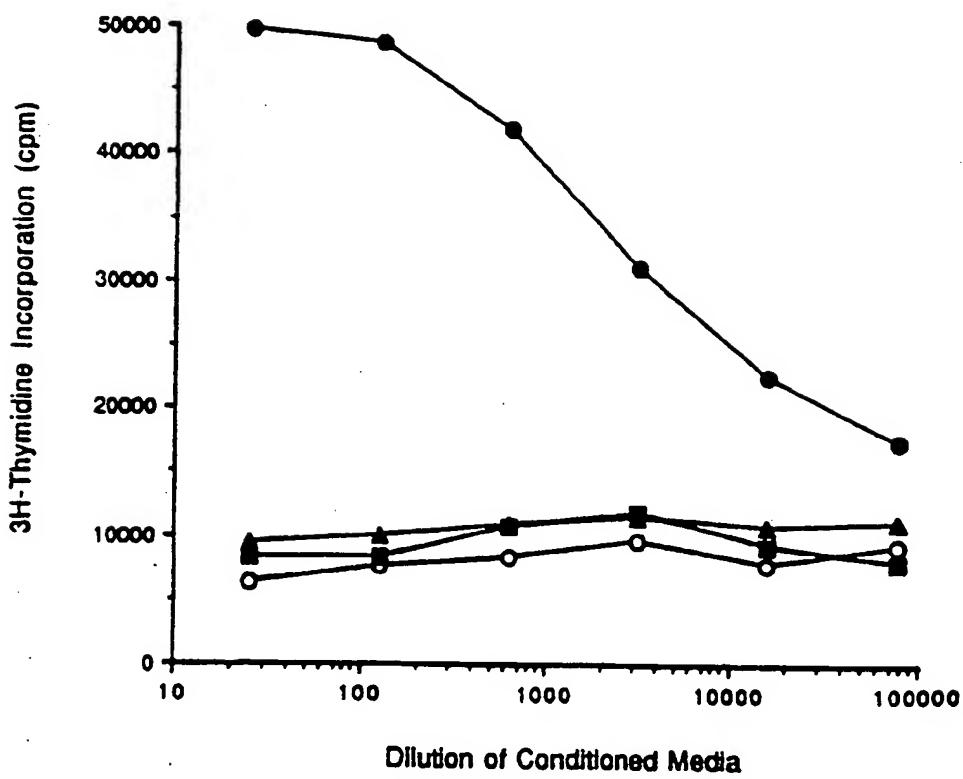
Fluorescence Intensity

EP 0 640 689 A2

Fig. 2

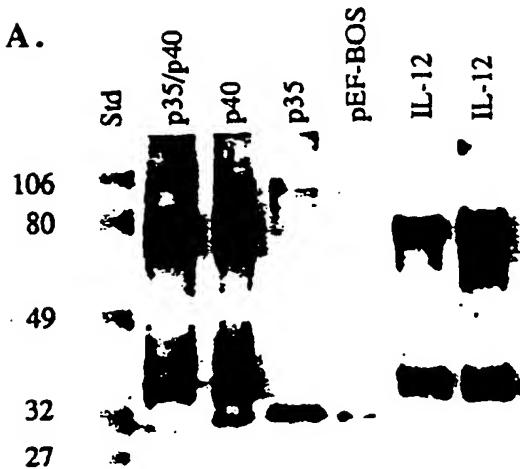
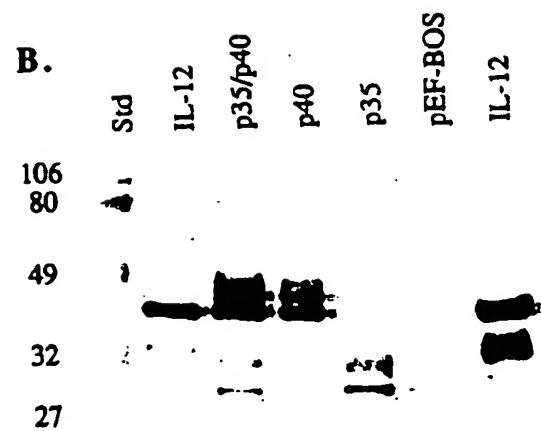


EP 0 640 689 A2

Fig. 3

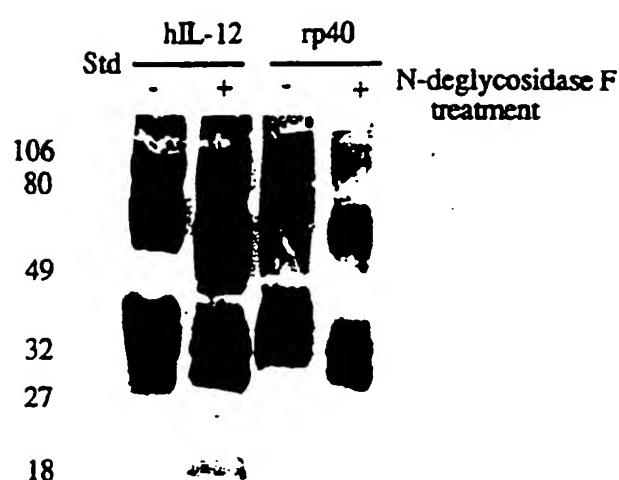
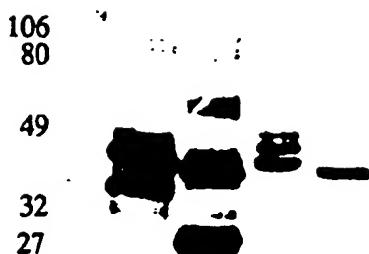
EP 0 640 689 A2

Fig. 4

A.**B.**

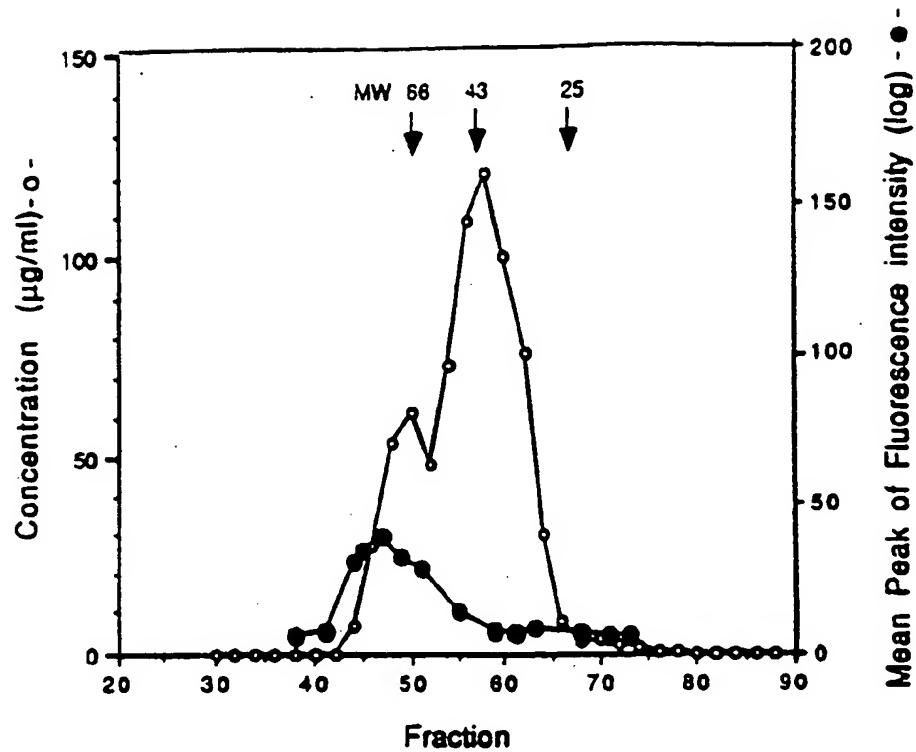
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Fig. 5

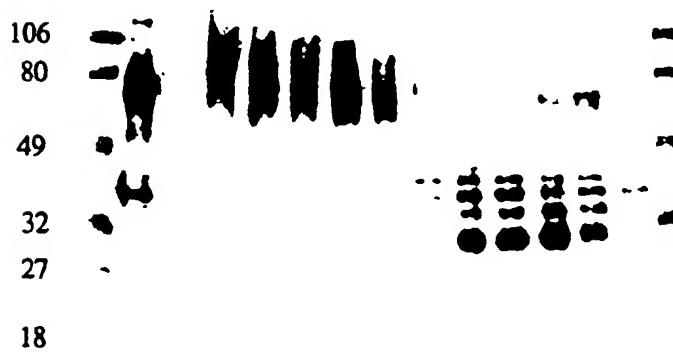
A.**B.**

EP 0 640 689 A2

Fig. 6

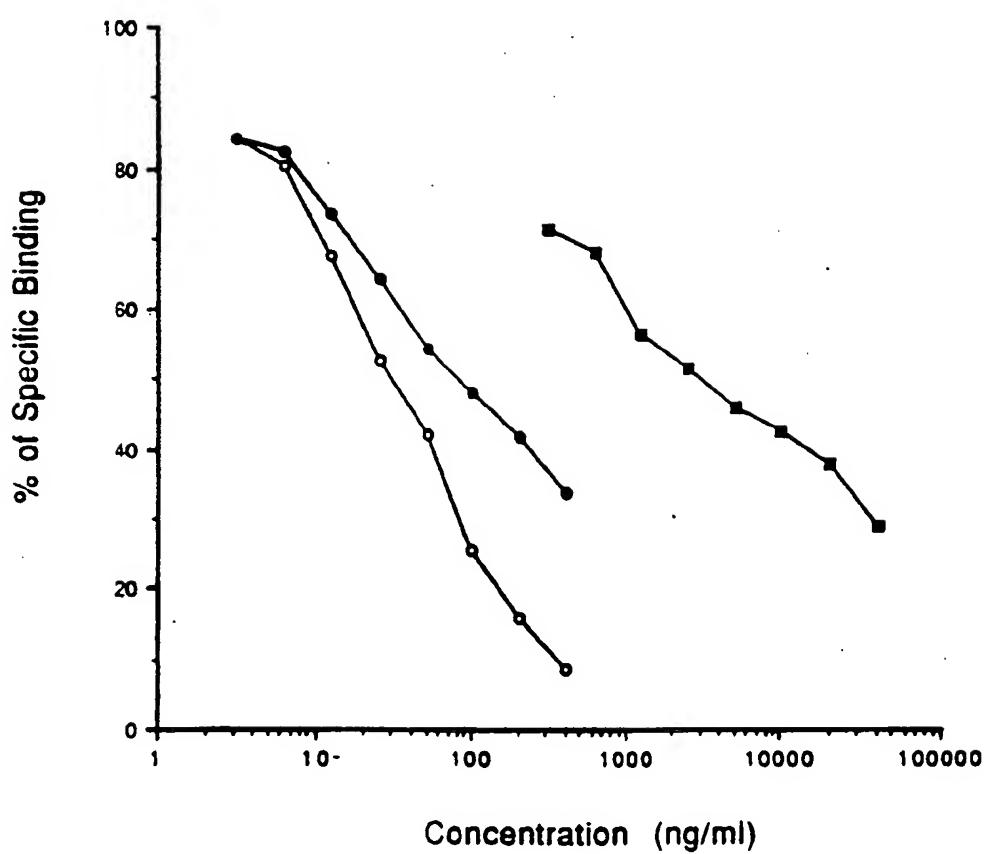


Std hIL-12 rp40 fractions



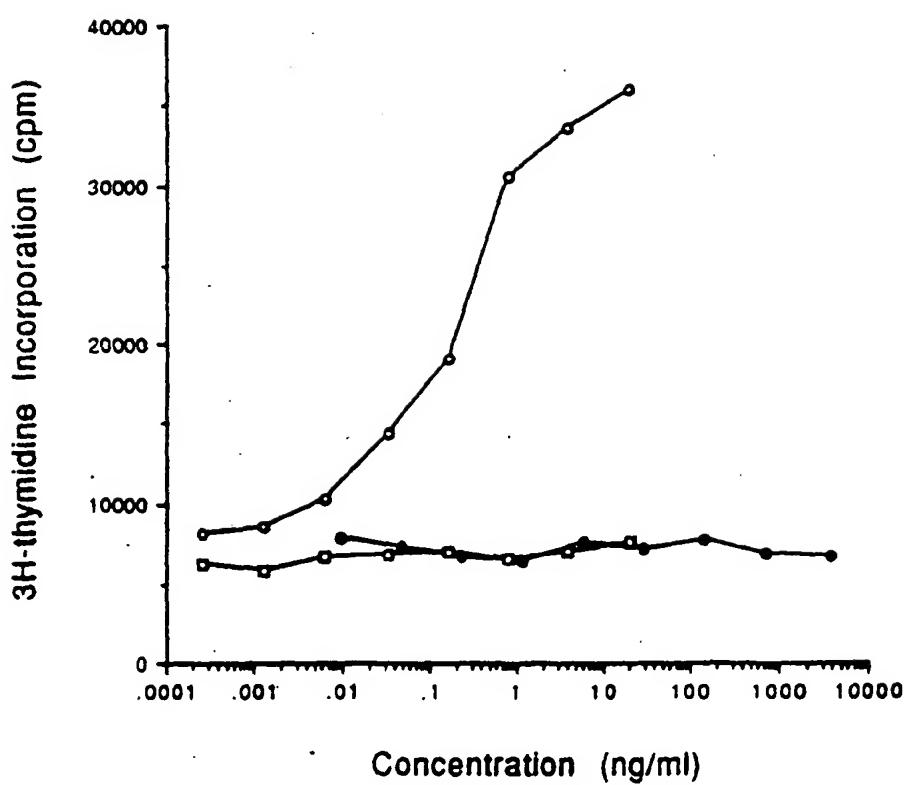
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Fig. 7



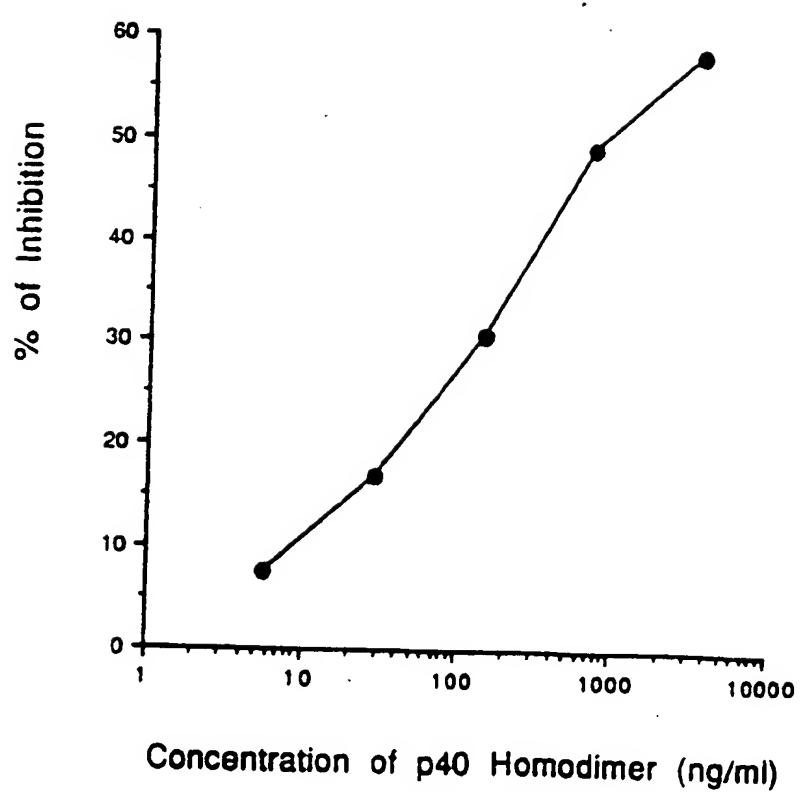
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Fig. 8



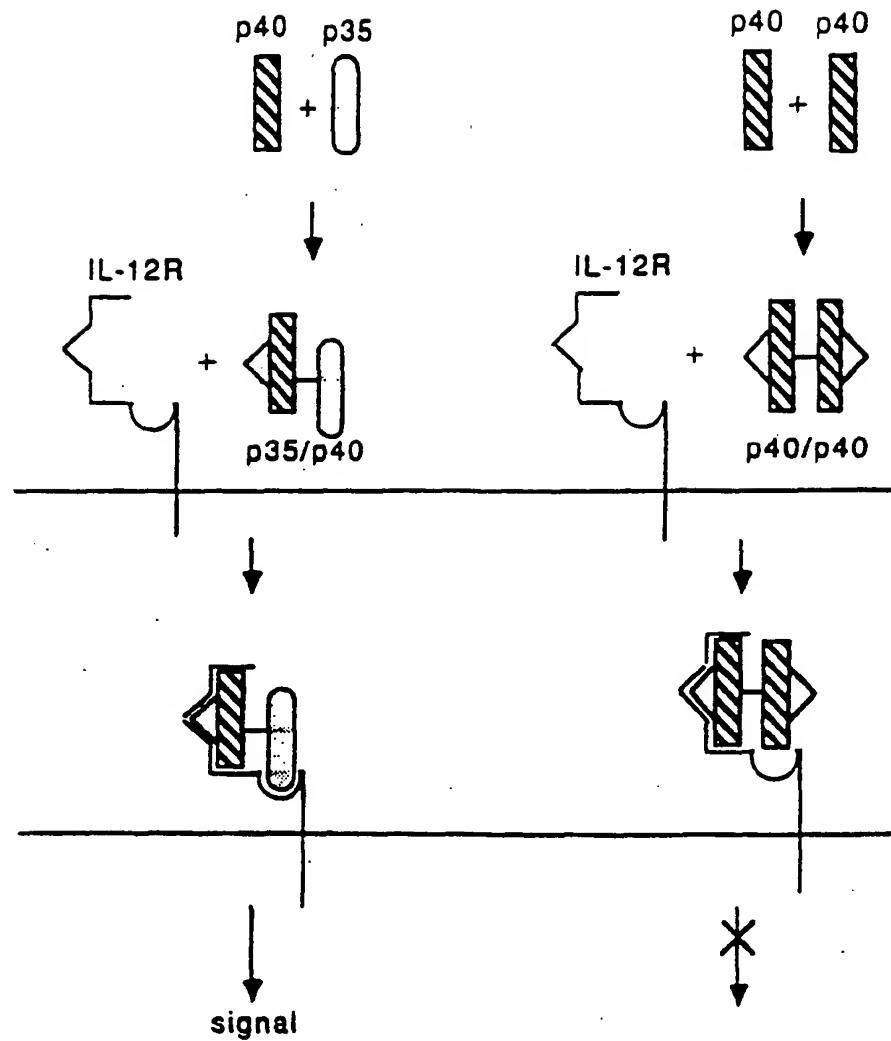
EP 0 640 689 A2

Fig. 9



EP 0 640 689 A2

Fig. 10

A.**B.**

Model of IL-12 Binding and Signalling